TOLTERODINE, A NEW MUSCARINIC RECEPTOR ANTAGONIST, IS METABOLIZED BY CYTOCHROMES P450 2D6 AND 3A IN HUMAN LIVER MICROSOMES

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ABSTRACT:

Tolterodine, a new muscarinic receptor antagonist, is metabolized via two pathways: oxidation of the 5-methyl group and dealkylation of the nitrogen. In an attempt to identify the specific cytochrome P450 enzymes involved in the metabolic pathway, tolterodine was incubated with microsomes from 10 different human liver samples where various cytochrome P450 activities had been rank ordered. Strong correlation was found between the formation of the 5-hydroxymethyl metabolite of tolterodine (5-HM) and CYP2D6 activity (r^2, 0.87), as well as between the formation of *N*-dealkylated tolterodine and CYP3A activity (r^2, 0.97). When tolterodine was incubated with human liver microsomes in the presence of compounds known to interact with different P450 isoforms, quinidine was found to be the strongest inhibitor of the formation of 5-HM.

Ketoconazole and troleandomycin were found to be the strongest inhibitors of the formation of *N*-dealkylated tolterodine. A weak inhibitory effect on the formation of *N*-dealkylated tolterodine was found with sulfaphenazole, whereas tranylcypromine did not inhibit the formation of this metabolite. Microsomes from cells overexpressing CYP2D6 formed 5-HM, whereas *N*-dealkylated tolterodine was formed by microsomes expressing CYP2C9, -2C19, and -3A4. The K_m for formation of *N*-dealkylated tolterodine by CYP3A4 was similar to that obtained in human liver microsomes and higher for CYP2C9 and -2C19. We conclude from these studies that the formation of 5-HM is catalyzed by CYP2D6 and that the formation of *N*-dealkylated tolterodine is predominantly catalyzed by CYP3A isoenzymes in human liver microsomes.

Tolterodine [(*R*)-*N*,*N*-diisopropyl-3-(2-hydroxy-5-methylphenyl)-phenylpropanamine] is a new muscarinic receptor antagonist specifically developed for the treatment of urinary urge incontinence and other symptoms associated with overactive bladder (Nilvebrant *et al.*, 1997). Following oral administration, tolterodine is rapidly absorbed from the gastrointestinal tract and exhibits extensive first-pass metabolism. Metabolites are formed via two pathways: oxidation of the 5-methyl group to a 5-hydroxymethyl derivative (5-HM[1]) (PNU-200577; labcode DD 01) and dealkylation of the nitrogen (fig. 1). In humans, about 80% of an administered oral dose of tolterodine is excreted in the urine, the main metabolites being the 5-carboxylic acids of tolterodine, *N*-dealkylated tolterodine, and their glucuronide conjugates. Less than 1% of the parent compound is excreted unchanged (Brynne *et al.*, 1997).

In a previous study in healthy volunteers, one subject showed notably lower systemic clearance than the overall average. This dissimilarity was probably due to differences in metabolic capacity (Brynne *et al.*, 1997). However, the specific P450 enzymes involved in the metabolism of tolterodine have not been identified. Such knowledge is of great importance to predict potential drug interactions and genetic variations in drug metabolism. In this study, we performed experiments in which the formation of metabolites of tolterodine was correlated with marker P450 activities in human liver mi-

[1] Abbreviations used are: P450, cytochrome P450; 5-HM, 5-hydroxymethyl metabolite of tolterodine; HPLC, high pressure liquid chromatography; Cl_i, intrinsic clearance (V_{max}/K_m); V, rate of metabolite formation.

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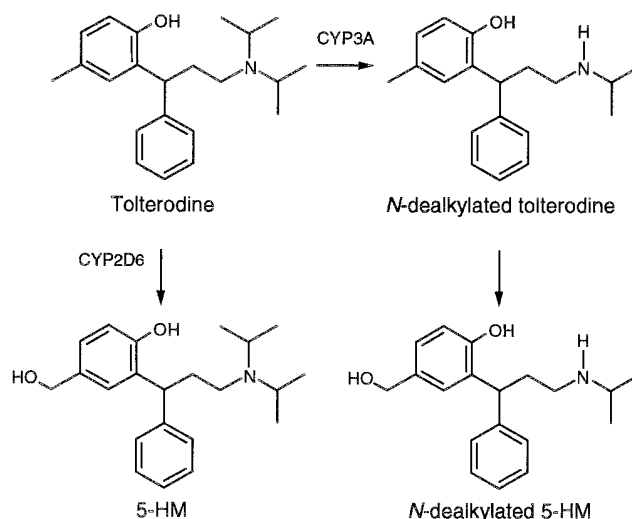

FIG. 1. *Main metabolic pathways of tolterodine in human liver microsomes.*

crosomes. We also used inhibitors and isoenzymes expressed using recombinant technology to determine the individual enzymes involved in the metabolism of tolterodine.

Materials and Methods

Chemicals. [^{14}C]Tolterodine 4.2 and 0.85 MBq/mg, 5-HM [(*R*)-*N*,*N*-diisopropyl-3-(2-hydroxy-5-hydroxymethylphenyl)-phenylpropanamine], *N*-dealkylated tolterodine, and *N*-dealkylated 5-HM were synthesized at Pharmacia & Upjohn AB (Uppsala, Sweden). β-NADPH was obtained from Merck KGaA (Darmstadt, Germany), whereas α-naphthoflavone, quinidine, sulfaphenazole,

tranylcypromine, and troleandomycin were obtained from Sigma. Ketoconazole was kindly provided by the Janssen Research Foundation (Beerse, Belgium). All other chemicals were of high purity and were obtained from usual commercial sources.

Human Liver Microsomes. A HepatoScreen test kit with 10 different human liver microsomal samples was obtained from Human Biologics, Inc. (Phoenix, AZ) and used in correlation experiments. Four of the samples were from males. The microsomes had been characterized with respect to the following enzyme activities: 7-ethoxyresorufin *O*-dealkylation (CYP1A), caffeine 3-demethylation (CYP1A2), coumarin 7-hydroxylation (CYP2A6), tolbutamide methyl-hydroxylation (CYP2C9), *S*-mephenytoin 4-hydroxylation (CYP2C19), dextromethorphan *O*-demethylation (CYP2D6), chlorzoxazone 6-hydroxylation (CYP2E1), testosterone 6β-hydroxylation (CYP3A), lauric acid 11-hydroxylation (CYP2E1) and lauric acid 12-hydroxylation (CYP4A), benzphetamine *N*-demethylation (unknown), and total P450 content. Pooled human liver microsomes were obtained from XenoTech LLC (Kansas City, KS) and used in enzyme kinetic and inhibition experiments.

Overexpressed P450 Isoenzymes. Microsomes containing expressed P450 isoforms from human lymphoblast cells (CYP1A1 and -1A2) or insect cells (CYP2C8, -2C9-Arg, -2C19, -2D6, and -3A4) and nontransfected cells were purchased from Gentest (Woburn, MA).

Incubation Conditions. All incubations with liver microsomes were carried out at a protein concentration corresponding to 1 mg/ml in 100 mM potassium phosphate buffer (pH 7.4) and 1 mM β-NADPH at 37°C. In the experiments measuring the enzyme kinetics, 5–200 μM tolterodine was incubated in a final volume of 250 μl for 15 min. In correlation experiments, the HepatoScreen test kit was incubated at 150 μM concentration in a final volume of 1 ml for 30 min. Inhibition experiments were carried out at tolterodine concentrations equal to the apparent K_m for formation of 5-HM and *N*-dealkylated tolterodine (7 and 50 μM, respectively) in a final volume of 1 ml for 15 min. The different inhibitors were dissolved in methanol and then added in 10 μl (1% v/v final concentration) to the microsomes (10 μl of methanol was used in control experiments). α-Naphthoflavone, sulfaphenazole, tranylcypromine, and troleandomycin were added at a final concentration of 1, 10, and 50 μM, quinidine at 0.1, 1, and 10 μM (5-HM) or at 1, 10, and 50 μM (*N*-dealkylated tolterodine), and ketoconazole at 0.01, 0.1, and 1 μM. Incubations containing troleandomycin were preincubated with microsomes and 1 mM β-NADPH for 15 min before addition of tolterodine. All other substances tested for inhibition were added just before tolterodine.

Microsomes containing expressed P450 isoforms from human lymphoblast cells or insect cells (20 pmol, respectively) were incubated with 10 μM tolterodine, 100 mM potassium phosphate buffer (pH 7.4) and 1 mM β-NADPH in a final volume of 250 μl at 37°C for 20 min. Microsomes from nontransfected cells were used as controls. In enzyme kinetic experiments, incubations were performed as described above with 10–200 μM tolterodine.

Under the conditions used, the formation of tolterodine metabolites was linear with respect to incubation time and protein concentrations.

The reactions were terminated by addition of acetone (1:1 v/v) and stored at −20°C. Before analysis, the microsomal protein was precipitated by centrifugation at 3200 rpm, and the acetone in the collected supernatant evaporated with a stream of nitrogen. A 100–200-μl aliquot of the remaining supernatant was used for HPLC analysis.

HPLC Analysis. The incubations were analyzed for the parent drug and its metabolites by HPLC using two LKB 2150 pumps, an LKB 2152 LC controller, Pharmacia UV-M monitor set at 280 nm, Beckman 171 radioisotope detector, a Supelco PKB 100 (2 cm) precolumn, and a Supelco PKB 100 (150 × 4.5 mm) column. The mobile phase was 20 mM ammonium acetate in methanol (pH 4.5). The solvent flow rate was 1 ml/min, and a gradient of decreasing polarity [time (min)/% methanol: 0/10, 5/20, 35/45, 40/100, 50/100) was used.

Calculations. The amount of each metabolite was calculated from the radiochromatogram as the (% area of the metabolite)/(% area of all metabolites + parent compound), and the results are expressed as in relation to milligram of microsomal protein or picomole of P450 per minute. The retention times of formed metabolites were compared with the retention times of synthesized reference standards, and their identity was further confirmed by electrospray ionization mass spectrometry. The enzymatic constants were calculated using
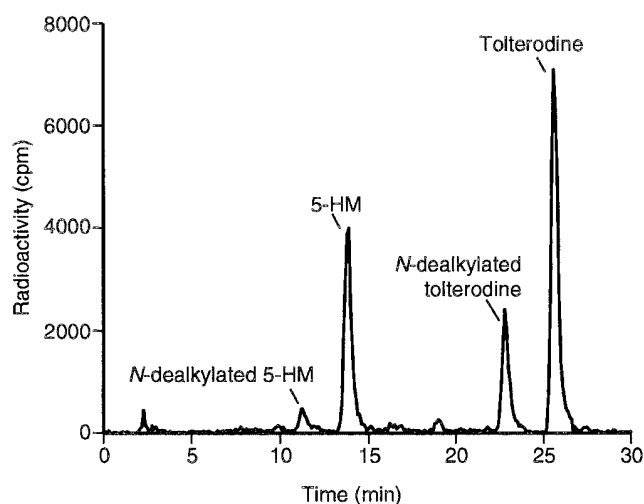

FIG. 2. *A representative radiochromatogram from incubation of tolterodine with human liver microsomes.*

nonlinear regression and correlation coefficients using linear regression and GraphPad Prism software.

Results

Metabolite Identification. Human liver microsomes converted [^{14}C]tolterodine into several products in the presence of β-NADPH. Fig. 2 shows a representative radiochromatogram containing four major peaks at retention times of 11.5, 14, 23, and 26 min. The identity of the metabolites was confirmed by comparison with the retention times and product-ion mass spectra, obtained by collision-induced dissociation of protonated molecular ions ([M+H]$^+$), of synthesized reference standards (data not shown). *N*-Dealkylated 5-HM (retention time, 11.5 min) had a [M+H]$^+$ ion at *m/z* 300, 5-HM (retention time, 14 min) at *m/z* 342, and *N*-dealkylated tolterodine (retention time, 23 min) at *m/z* 284. The peak at 26 min corresponded to intact [^{14}C]tolterodine.

Enzyme Kinetics. Pooled human liver microsomes were used to calculate the apparent K_m, V_{max}, and Cl_i values, which are shown in table 1. Fig. 3 shows Eadie-Hofstee plots for the formation of 5-HM and *N*-dealkylated tolterodine. Plots for both metabolites were linear, indicating the involvement of single P450 isoforms in the reactions.

Correlation Experiment. Tolterodine was incubated with ten different human liver microsomal samples, where different P450 activities had been rank ordered (HepatoScreen test kit). The rate of formation of 5-HM and *N*-dealkylated tolterodine (fig. 1) varied approximately 6- and 7-fold, respectively, among the samples (fig. 4). Correlations between the rate of formation of tolterodine metabolites and different P450 marker activities are shown in table 2. The formation of 5-HM correlated strongly with dextromethorphan *O*-demethylation (r^2, 0.87), a marker for CYP2D6 activity (Schmid *et al.*, 1985), whereas no correlation was obtained for other P450 activities. Testosterone 6β-hydroxylation, a marker for CYP3A activity (Waxman *et al.*, 1988), showed a strong correlation with the formation of *N*-dealkylated tolterodine (r^2, 0.97). A relatively strong correlation was also obtained toward total P450 content and the formation of this metabolite (r^2, 0.76).

Chemical Inhibition Experiments. The effect of various substances, at different concentrations, on the formation of metabolites in pooled human liver samples is shown in fig. 5. α-Naphthoflavone, an inhibitor of CYP1A isoenzymes (Guengerich, 1992; Tassaneeyakul *et*

TABLE 1

Enzyme kinetics for the formation of the 5-hydroxymethyl and N-dealkylated metabolites of tolterodine in pooled human liver microsomes

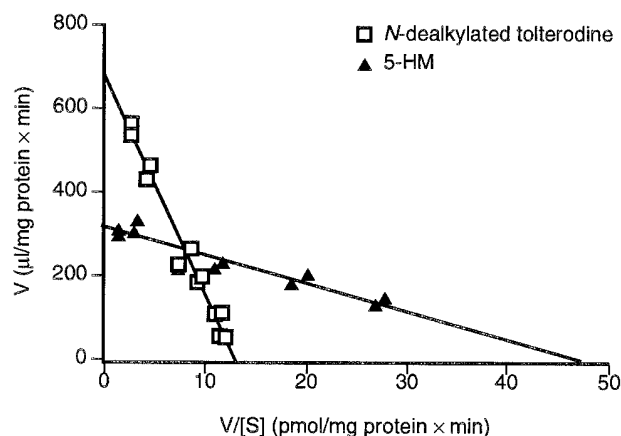| Parameter	Metabolite	
	5-HM	N-Dealkylated Tolterodine
V_{max} (pmol/mg protein $\times$ min)	317	687
K_m (μM)	7	52
CI_i (μl/min $\times$ mg protein)	47	13

FIG. 3. *Eadie-Hofstee plots for the formation of the 5-HM and N-dealkylated tolterodine in human liver microsomes ([S], substrate concentration).*
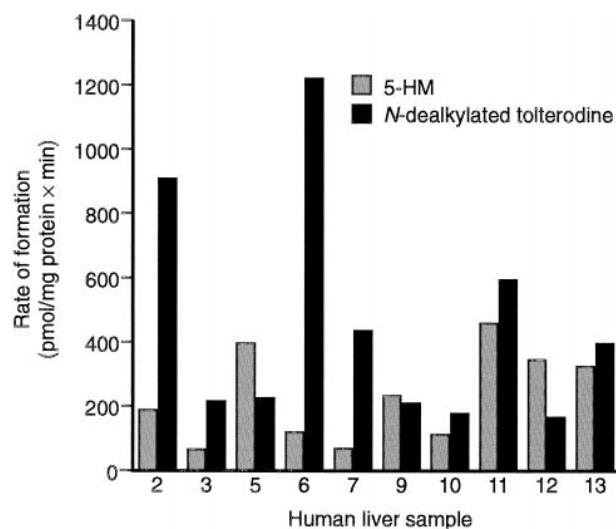

FIG. 4. *Rate of formation of the 5-HM and N-dealkylated tolterodine in human liver microsomes from different individuals.*

TABLE 2

Correlation between the rate of formation of tolterodine metabolites and total P450 content and different P450 marker activities in human liver samples

Metabolism	P450 Isoenzyme	Correlation (r^2)	
		5-HM	N-Dealkylated Tolterodine
Total P450 content		—[a]	0.76[c]
7-Ethoxyresorufin O-dealkylation	1A	—[a]	—[a]
Caffeine 3-demethylation	1A2	—[a]	—[a]
Coumarin 7-hydroxylation	2A6	—[a]	—[a]
Tolbutamide methyl-hydroxylation	2C9	—[a]	0.64[d]
S-Mephenytoin 4-hydroxylation	2C19	—[a]	—[a]
Dextromethorphan O-demethylation	2D6	0.87[b]	—[a]
Chlorzoxazone 6-hydroxylation	2E1	—[a]	0.22
Testosterone 6β-hydroxylation	3A	—[a]	0.97[b]
Lauric acid 11-hydroxylation	2E1	—[a]	0.29
Lauric acid 12-hydroxylation	4A	—[a]	0.43
Benzphetamine N-demethylation	Unknown	—[a]	0.27

[a] $p > 0.2$.
[b] $p < 0.0001$.
[c] $p < 0.001$.
[d] $p < 0.01$.

FIG. 5. *Effect of various cytochrome P450 inhibitors on the rate of formation of the 5-HM (A) and N-dealkylated tolterodine (B) at a 7 and 50 μM concentration of tolterodine, respectively, in human liver microsomes.*

al., 1993), did not significantly inhibit the metabolism of tolterodine at concentrations used in this study. Quinidine, which is regarded as a specific inhibitor of CYP2D6 (Inaba *et al.*, 1985), almost completely inhibited the formation of 5-HM at 10 μM. High concentrations of quinidine also produced slight inhibition of the formation of N-dealkylated tolterodine. The strongest inhibition of the formation of N-dealkylated tolterodine was observed with ketoconazole, which inhibited formation of this metabolite by >70% at a concentration of 1 μM but did not affect the formation of 5-HM. A strong inhibition was also observed for the formation of N-dealkylated tolterodine with troleandomycin, which is known to specifically interact with CYP3A isoenzymes (Guengerich and Shimada, 1991). Troleandomycin had
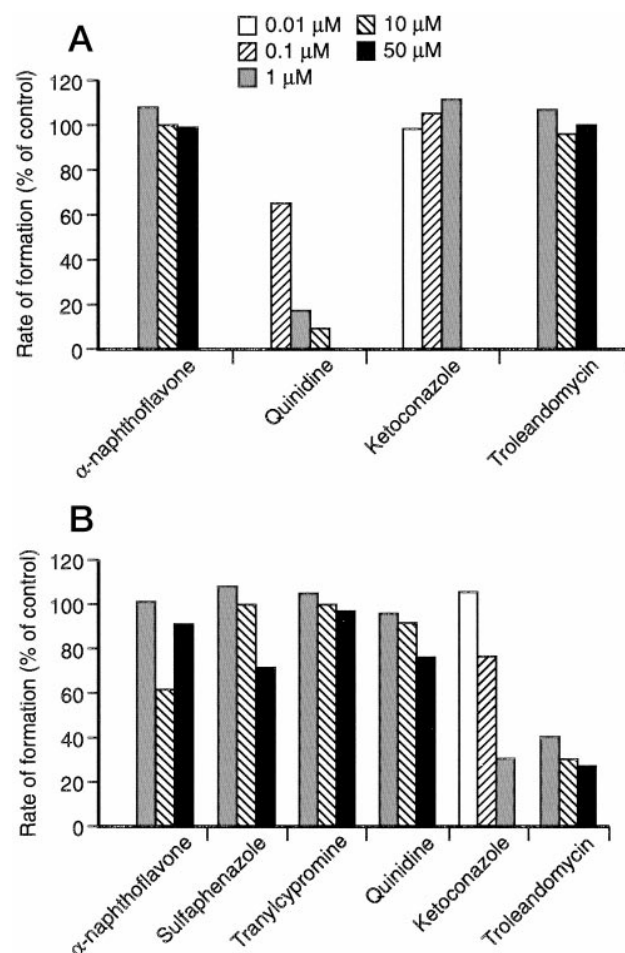
no effect on the formation of 5-HM. Sulfaphenazole, a competitive inhibitor of CYP2C9 (Baldwin *et al.*, 1995), was found to have a weak inhibitory effect on the formation of N-dealkylated tolterodine at a concentration of 50 μM (<30%), whereas tranylcypromine, an inhib-

TABLE 3

Metabolism of tolterodine by microsomes from cells overexpressing P450 isoenzymes

P450 Isoenzyme	Rate of Formation (pmol/pmol P450 × min)	
	5-HM	*N*-Dealkylated Tolterodine
1A1	ND	ND
1A2	ND	ND
2C8	ND	ND
2C9-Arg	ND	0.25 ± 0.05
2C19	ND	1.51 ± 0.06
2D6	5.0 ± 0.35	ND
3A4	ND	0.23 ± 0.03

Data are mean ± SD of two experiments. ND, not detectable.

TABLE 4

Enzyme kinetics for the formation of N-dealkylated tolterodine in microsomes from cells overexpressing P450 2C9-Arg, -2C19, and -3A4

Parameter	P450 Isoenzyme		
	2C9-Arg	2C19	3A4
V_{max} (pmol/pmol P450 × min)	1.86	12.3	1.48
K_m (µM)	74	70	47
Cl_i (µl/min × pmol P450)	0.025	0.176	0.031

itor of CYP2C19 (Inaba *et al.*, 1985; Wienkers *et al.*, 1996), did not affect the formation of this metabolite at concentrations used in this study.

Metabolism by Overexpressed P450 Isoenzymes. Microsomes from cells overexpressing CYP1A1, -1A2, -2C8, -2C9-Arg, -2C19, -2D6, or -3A4, together with microsomes from nontransfected cells, were incubated with 10 µM tolterodine in the presence of β-NADPH. Microsomes from cells overexpressing CYP2D6 catalyzed the formation of 5-HM, whereas *N*-dealkylated tolterodine was formed in microsomes overexpressing CYP2C9-Arg, -2C19, and -3A4 (table 3). The enzyme kinetic constants for the formation of *N*-dealkylated tolterodine by microsomes are shown in table 4. No metabolites were formed in incubations with microsomes from nontransfected cells (data not shown).

Discussion

The results presented in this study provide evidence for the involvement of CYP2D6 in the formation of 5-HM and CYP3A as the major enzyme involved in the formation of *N*-dealkylated tolterodine. The formation of 5-HM is dependent on CYP2D6, based on a high correlation with dextromethorphan *O*-demethylation, inhibition by quinidine, and formation of the metabolite only in cell microsomes overexpressing CYP2D6. The formation of *N*-dealkylated tolterodine correlated strongly with testosterone 6β-hydroxylation. A relatively strong correlation was also obtained toward total P450 content. This is in accordance with immunochemical and inhibition studies indicating that as much as 60% of the total P450 content in human liver may be CYP3A isoenzymes (Guengerich, 1990).

According to the results from the HepatoScreen test kit, the involvement of CYP2C9 in the formation of *N*-dealkylated tolterodine could be substantial. This was, however, not confirmed by experiments with sulfaphenazole, an inhibitor described as specific for this P450 isoenzyme (Baldwin *et al.*, 1995). Only high concentrations (50 µM) showed a weak inhibitory effect (<30%) on the formation of *N*-dealkylated tolterodine. Furthermore, the activities of CYP2C9 and -3A in the test kit were found to correlate with each other (r^2, 0.60). Tranylcypromine, which has been described as an inhibitor of *S*-mephenytoin 4-hydroxylation (CYP2C19) (Inaba *et al.*, 1985; Wienkers *et al.*, 1996), did not inhibit the formation of *N*-dealkylated tolterodine at concentrations used in this study. Ketoconazole and troleandomycin, both known to interact with CYP3A (Guengerich and Shimada, 1991; Maurice *et al.*, 1992), were found to be the strongest inhibitors of the formation of *N*-dealkylated tolterodine. A marginal inhibitory effect on the formation of this metabolite was also observed at high concentrations of quinidine. This is probably not an effect on CYP2D6 but rather a result of nonspecific inhibition of CYP3A, as quinidine is a known substrate for this P450 isoenzyme (Guengerich *et al.*, 1986). The formation of *N*-dealkylated tolterodine was detected

in microsomes overexpressing not only CYP3A4 but also CYP2C9 and -2C19. The K_m for formation of *N*-dealkylated tolterodine with CYP3A4 was 47 µM, similar to that obtained in human liver microsomes (52 µM), whereas K_m values for CYP2C9 and -2C19 was about 70 µM. Estimates of the relative Cl_i values per picomole of P450 using these overexpressed enzymes indicated that formation of *N*-dealkylated tolterodine was about 6- and 7-fold higher for CYP2C19, respectively, compared with CYP3A4 and -2C9. However, human liver does not contain equimolar concentrations of the different P450 isoforms, and the levels of β-NADPH-P450 reductase may vary considerably among different cDNA-expressed preparations and also differ from that found in human liver. Consequently, enzyme turnover numbers may vary substantially not only between the different preparations but also in comparison to human liver. Thus, there is no firm basis for extrapolation of relative Cl_i values obtained with cDNA-expressed enzymes to human liver. CYP3A4 had the lowest K_m compared with CYP2C9 and -2C19. As CYP3A4 is also the major P450 isoenzyme expressed in human liver, it is reasonable to assume that the V_{max} in human liver microsomes is higher for CYP3A4 than for CYP2C9 and -2C19. If this is the case, then the contribution of lower V_{max}, higher K_m enzymes, *i.e.* CYP2C9 and 2C19, will be completely obscured. Taken together, the results of the present study indicate that the formation of *N*-dealkylated tolterodine is predominantly catalyzed by CYP3A4 in human liver microsomes. Estimates of the Cl_i values from data obtained in pooled human liver microsomes also showed good agreement with the results of a study in healthy volunteers that reported that about 80% of tolterodine is predominantly metabolized via formation of 5-HM (Brynne *et al.*, 1997).

Clinical studies have demonstrated that individuals with reduced CYP2D6-mediated metabolism represent a high-risk group in the population with a propensity to develop adverse drug effects (Smith, 1986). The number of drugs identified as being affected by CYP2D6 polymorphism has increased steadily over the years and includes diverse classes such as β-adrenoreceptor antagonists, tricyclic antidepressants, neuroleptics, and other miscellaneous drugs like dextromethorphan and codeine (Daly *et al.*, 1993; Murray, 1992). CYP3A is the major P450 subfamily in human liver and is involved in the metabolism of >50% of pharmaceutical drugs on the market. In addition, CYP3A enzymes have been reported to be involved in interactions with several drugs such as macrolides, ketoconazole, cyclosporin, and others (Honig *et al.*, 1993; Periti *et al.*, 1992; Pichard *et al.*, 1990; Wrighton and Stevens, 1992). The possibility of clinical drug interaction at the enzyme level thus exists, especially if tolterodine is administered at the same time as a compound that is preferentially metabolized by CYP2D6 or to individuals associated with the CYP2D6 poor metabolizer phenotype. However, the large amount of CYP3A in the liver and the fact that tolterodine is predominantly eliminated via oxidation by CYP2D6 makes it less likely that clinically significant drug-drug interactions would occur with CYP3A substrates in individuals with the CYP2D6 extensive metabolizer phenotype.

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